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Bone

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Sclerostin Antibody Treatment Improves Fracture Outcomes in a Type I Diabetic Mouse Model

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Abstract: Type 1 diabetes mellitus (T1DM) patients are at higher risk of osteopenia and impaired fracture healing due to decreased osteoblast activity. Further, no adequate treatments are currently available that can restore impaired healing in T1DM; hence a significant need exists to investigate new therapeutics for treating orthopedic complications. Sclerostin (*Sost*), a Wnt antagonist, negatively regulates bone formation. We hypothesized that Sost antibody (SostAb) treatment in Streptozotocin (*STZ*)-induced T1DM mice would improve impaired fracture *via* elevated Wnt signaling to rescue the lack of osteoblast activity. After fracture, SostAb was administered twice weekly up to 21 days post-fracture, and microscale computed tomography analysis of intact bones and calluses at 21- (11 weeks of age) and 42- days (14 weeks of age) post fracture showed decreased trabecular and cortical bone parameters in *STZ* mice compared to *Controls*. SostAb-treated groups showed improved bone parameters that continued for 3 weeks after cessation of antibody treatment. Markers of osteoblast maturity such as Runx2, Collagen I, Osteocalcin, and DMP1 were reduced, while an abundant number of Sp7/Osterix-positive early osteoblasts were seen on the bone surface of *STZ* calluses. These results suggested that *STZ* calluses have poor osteogenesis resulting from failure of osteoblasts to fully differentiate and produce mineralized matrix, which resulted in less mineralized bone and callus. SostAb treatment enhanced fracture healing in both normal and *STZ* groups, and in *STZ+SostAb* mice, also reversed the lower mineralization seen in S*TZ* calluses. Histomorphometry of calluses revealed that although the bone volume: total volume ratio was improved with SostAb treatment, the bone was less mineralized than *Controls*. Sclerostin and activated β-Catenin levels were examined, and Sclerostin was elevated in *STZ* mice and β-Catenin activity was reduced. SostAb treatment resulted in elevated β-Catenin activity, but also dramatically increased of the levels of Sclerostin protein in bone and in circulation. Our results suggest that SostAb treatment rescues the impaired osteogenesis seen in this T1DM fracture model.

KEY WORDS:
Sclerostin, Type I Diabetes, fracture repair, Streptozotocin, STZ, osteoblast differentiation, Sclerostin antibody

Introduction

Type 1 diabetes mellitus (T1DM) patients have delayed or impaired fracture healing (Graves, Alblowi, Paglia, O'Connor, & Lin, 2011) as a result of osteopenia (Hamilton et al., 2009; McCabe, 2007; Hofbauer et al., 2007) along with an increased fracture risk (Nyman, et al., 2011) (Schwartz A. , 2003) and risk of osteoporosis with age. Fracture risk increases with age, as well as with complications from diabetes, such as kidney disease, and length of time after diagnosis (Vestergaard et al., 2009) Recent studies revealed that the low bone mass phenotype is caused by reduced osteoblast activity (Coe et al., 2010; McCabe 2007). Although some studies show association of T1DM and increased

osteoclast activity, many studies fail to show any change in resorption (Hamilton et al., 2009). In contrast to the osteopenia observed in T1DM patients, some type 2 diabetes mellitus (T2DM) patients have an increase in bone mineral density and high bone mass phenotype (de Liefde et al., 2005) (Issa, Zantout, & Azar, 2011). However, T2DM patients also appear to have impaired fracture healing, which indicates abnormal bone formation or remodeling (de Liefde et al., 2005) (Shu, et al., 2012).

Glucose control, although important for treatment, does not provide universal protection from downstream effects of diabetes. The attachment of glucose moieties (glycation) that interferes with protein and tissue structure and function can be reduced but not prevented with close glucose control (Ceriello, 2012). Glycation of type I collagen in bone reduces the ability of osteoblasts to adhere to the matrix, reduces alkaline phosphatase (bone-forming) activity. In type I diabetic rats, the healing phenotype of closed fractures showed a delay in healing time in both insulin- and blood glucose-controlled diabetes (Beam, et al., 2002), which is also true for human patients (Loder, 1988). In addition, oxidative stress in tissues leads to much of the destructive sequelae in diabetic patients, causing pancreatic beta-cell death, kidney disease, cardiovascular disease, and may contribute to the effects of diabetes seen in the skeleton. Restoration of normal glucose levels does not prevent oxidative stress in tissues (Erejuwa, 2012). Although insulin control of blood glucose levels is an integral part of T1DM treatment, it cannot completely recover the diabetic osteopenia (Campos Pastor et al., 2000), nor alleviate delayed fracture healing in T1DM; an additional therapeutic intervention is needed.

Wnts are a family of secreted proteins that are involved in many aspects of embryonic development including primary axis formation (Petersen and Reddien, 2009), kidney organogenesis (Halt and Vainino, 2014) and limb patterning and outgrowth (Church and Francis-West, 2002). Wnt ligand binding at the cell surface transduces a signal into the cell cytoplasm by binding to LRP5/6 and Frizzled co-receptors. β-catenin is activated and translocates to the nucleus where it binds to DNA, along with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, to activate downstream target genes. (Nusse R,2012). During inactivation or inhibition of canonical Wnt signaling, GSK3β, a component of the destruction complex, phosphorylates β–catenin for degradation by proteasomes, and so is a key regulator of β-catenin activity. Canonical Wnt signaling has been previously demonstrated to promote bone formation in genetically modified animals. For example, loss of function of LRP5 results in an osteopenic phenotype (Kato et al., 2002; Cui et al, 2011), while other mutations in LRP5 results in a high bone mass phenotype in rodents and humans (Little et al., 2002; Babij et al., 2003) revealing the direct involvement that Wnts have in bone formation. Genetic inhibition of GSK3β increases Wnt signaling through β-catenin, and enhances bone formation and repair (Arioka et al., 2013).

Canonical Wnt signaling, which relies on β-catenin activity, is involved during fracture repair. (Chen, et al., 2007). Wnt ligand binding at the cell surface transduces a signal into the cell cytoplasm by binding to LRP5/6 co-receptors. β-catenin is activated and translocates to the nucleus where it binds to DNA, along with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, to activate downstream target genes. (Nusse R,2012). In TCF reporter mice, where a LacZ gene is placed downstream of tandem TCF binding sites, the reporter is only expressed upon activated β-catenin and TCF binding, thus providing a way to assess canonical Wnt signaling activity. After closed tibial fracture in mice, β-catenin is activated during early stages of repair, continues to be activated throughout the entire fracture repair time course (Chen, et al., 2007). This study also demonstrated that that β-catenin hyperactivity during fracture repair improves fracture healing even when β-catenin activity is increased in osteoblasts cells only (Chen, et al., 2007). In addition, β-catenin activation is a requirement for mesenchymal stem cell differentiation into osteoblasts, and a lack of β-catenin in mice results in decreased bone density (Day, et al., 2005). Furthermore, the lack of β-catenin in

osteoblast cells results in inhibition of osteoblast differentiation. Type 1 diabetic rats showed a decrease in Wnt downstream effectors, phosphorylated glycogen synthase kinase 3β (GSK3β) and activated β-catenin along with an increase in Sost protein level, resulting in decreased osteoblast activity (Hie et al., 2011). This suggests that the high blood glucose environment alters osteoblast activity *via* Wnt signaling. Therefore, influencing Wnt signaling to enhance osteoblast activity during fracture repair may be a potential therapeutic target to improve impaired healing in T1DM patients.

A potent Wnt antagonist, Sclerostin (Sost), is secreted by osteocytes (terminally-differentiated osteoblasts trapped within bone mineral matrix) and functions to inhibit bone formation (Li, et al., 2005). In humans, lack of Sclerostin causes sclerosteosis, a generalized skeletal hyperostosis disorder that results from elevated Wnt signaling/osteoblast activity (Brunkow et al., 2001; Balemans et al., 1999), while non-coding deletions of gene regulatory regions that control SOST expression result in similar phenotypes (Balemans et al., 2002; Collette et al., 2012). In animal models, overexpression of human *SOST* causes osteopenia and limb defects (Loots, et al., 2005; Collette et al., 2010), while lack of Sost in knockout mice causes 3-4 times more BMD, consistent with human phenotypes (Li et al., 2008; Collette, et al., 2012). Type 2 diabetic patients have elevated circulating SOST levels compared to non-diabetic patients (Gennari et al., 2012), suggesting that Wnt signaling is altered in diabetic patients and may contribute to the observed osteopenia and delayed healing phenotype. Sost antibodies (SostAb) have been shown to enhance bone healing in animal models and ovariectomized rats (PK et al., 2014) (McDonald et al., 2012) by increasing bone formation and mass due to enhanced osteoblast function, determined by increased osteocalcin protein, which is indicative of active, mature osteoblasts. SostAb treatment in type 2 diabetic rats has also been shown to improve bone mass and strength (Hamann et al., 2013), however this effect has not yet been examined in T1DM models.

In this study, we have administered Sost-neutralizing antibody *in vivo* to *STZ*-induced T1DM mice during fracture repair. By enhancing canonical Wnt signaling during fracture repair we have shown improved fracture repair and resolved osteopenia in T1DM mice. This effect was prolonged and improved bone quality even after treatment had been discontinued. We demonstrate for the first time that sclerostin antibodies counteract direct effects of high glucose-driven elevation of Sost levels in uncontrolled diabetes, indicating a therapeutic benefit of modulating Wnt signaling in T1DM patients.

Methods

Animals and Fracture Model

Male C57BL6/J mice at six weeks old were injected daily with Streptozotocin (*STZ)* (50mg/kg) or phosphate buffered saline (PBS) for 5 days. Prior to mid-femur fracture surgeries, blood glucose readings of ≥300 mg/dL, obtained one week after the last injection, confirmed diabetic status of *STZ*-treated mice. At eight weeks old, mid-femoral fractures were generated using a closed Einohorn model (Bonnarens and Einhorn, 1984). A 27G needle was surgically placed in the shaft of the femur and a dropped weight was used to induce a transverse fracture, and fracture placement was confirmed by radiography. Sost antibody, a sclerostin-neutralizing monoclonal antibody suitable for *in vivo* use in mice, (SostAb) (25 mg/kg), or PBS was administered subcutaneously twice weekly up to 21 days post-fracture or 11 weeks of age, for a total of five injections, (*Control*, *STZ*, *SostAb*, and *STZ+SostAb* groups, each also with age-matched, uninjured cohorts). Antibody or PBS treatment was then discontinued for three weeks until 42 days post-fracture, or 14 weeks of age. At 21 days and 42 days post-fracture, bones were dissected and processed for microscale-computed tomography (μCT), RNA, histology and immunofluorescence (IF). A schematic diagram of the treatment regimen is shown in Figure 1A. All animal work was performed under an IACUC-approved Animal Use Protocol at an AAALAC-accredited facility.

Histology and Immunofluorescent Staining

Collected tissues were fixed in 10% neutral buffer formalin for 72 hours and then decalcified in 0.5M EDTA, pH 7.3, at 4°C until completion, as determined by weight-loss-weight gain and radiographic confirmation. Tissues were infiltrated and embedded into paraffin blocks and sectioned (6µm) on glass slides and baked at 42°C overnight. Alcian Blue and Nuclear Fast Red stain on histological slides were first de-waxed and stained with Alcian Blue pH 1.5 (stains cartilage) and counterstained with nuclear fast red and mounted with Permount. For immunohistochemistry, Uni-trieve (Innovex) was used for the antigen retrieval for 30 minutes at 65°C, unless stated otherwise. Primary antibodies against Runx2 (abcam, ab76956), Collagen Type 1 (calbiochem 234167), Osteocalcin (abcam, ab10911), active Caspase 3 (cellsig 9661), were used. Anti-Sost (R&D, AF1589) required Trypsin/EDTA at 37°C for 25 minutes for antigen retrieval. Anti-Activated beta-catenin (Millipore, 8E7, 05-665) required Uni-trieve, Proteinase K (15µg/ml) for 15 minutes, and Rodent Block. Secondary antibodies (Alexa Fluor 488 (green) or 594 (red), Molecular Probes) were used or detection. Stained slides were mounted with Prolong Gold with DAPI (Molecular Probes). ImagePro Plus V7.0 and CCD QIClick camera were used for imaging.

Micro-CT

Fractured and uninjured age-matched hind-legs collected at 21 and 42 days post fracture were collected, fixed in 10% neutral buffer formalin for 72 hours at 4°C and stored in 95% ethanol until measured. Distal femur, callus, and L4 vertebrae were measured and analyzed for bone parameters (n=6-10 per group) (µCT 40, SCANCO, Brüttisellen, Switzerland) according to the guidelines for µCT analysis of rodent bone structure (Bouxsein et al., 2010): energy 55 kVp, intensity 114 mA, integration time 900 ms, 6 µm nominal voxel size. For fracture analysis, age-matched animals (11 or 14-weeks of age) were compared to 21 day or 42 day fractures for cortical measurements. The threshold for "bone" was set at 395, which is approximately equal to 650 m HA/cm^3. Callus volume (TV) was measured including only callus that was outside of the native femur, and excluding the native bone volume of the original cortical bone and marrow space.

RNA

Control and *STZ* femora at 11 weeks of age were dissected free of adherent tissue and epiphyses were removed. Femoral cortices were centrifuged briefly to separate the bone marrow. The cortical bone was immediately homogenized with Qiazol. Qiagen RNeasy microarray kit was used to extract the RNA from the cortical bone and marrow and optical density and curve analysis were used (Nanodrop 2000) for quality verification (n=3 per group).

Immunosorbent Assay

During dissections, serum was collected in serum collection tubes (BD microtainer). After separation, serum was stored in -20°C until use. A mouse sclerostin ELISA kit (ALPCO, 41-SCLMS-E01) was used according to the manufacturer's instructions to measure serum sclerostin levels. Data is represented as mean +/- standard deviation (n=5 per group).

Cell Culture

UMR-106 cells were used to examine short-term effects of a high glucose environment. Cells were cultured on tissue-cultured treated plastic. Cells were plated at 20% confluence, in α-MEM media containing 5% fetal bovine serum and 5% bovine calf serum, antibiotic-antimycotic was added. Cells were subjected to normal (5.5 mM) or high (25 mM) D-glucose. Cells were treated until 80% confluent (48 hours) and RNA was immediately extracted by adding Qiazol to each well and proceeding as above for bone samples.

Quantitative PCR

(to obtain info from Nick, pending results)

Statistics
All data were expressed as the mean ± standard deviation. For statistical analysis, *Student's T-test* with a two-tailed distribution was used, with two-sample equal variance (homoscedastic test), for significance. p <0.05 was considered significant.

Results
SostAb improves the bone loss phenotype in STZ mice
STZ was used to induce a type I diabetic state in mice at 6 weeks of age. At 8 weeks of age, fractures were generated; age-matched and treatment-matched uninjured controls were also used. *SostAb* and *STZ+SostAb* groups were given 5 doses of *SostAb,* and at 21 days (or 11 weeks of age) or 42 days after inury (14 weeks of age), samples were collected for analysis (Fig. *1A)*. At 11 weeks of age, *STZ*-treated mice *(STZ)* showed abnormal growth plates, with reduced trabecular bone and less organized cartilage matrix deposition in the growth plate (Fig. *1D*, yellow arrowheads), compared to *Controls* (Fig. *1B, D*). We also noted an increase in adipocytes in the bone marrow of *STZ*-treated mice, (Fig. *1D,H,* black arrowheads), as well as reduced trabecular bone (Fig. *1F, H*), supporting previous reports of bone loss in *STZ*-treated animals (Hamada et al., 2007; Fowlkes et al., 2013). All groups showed increased trabecular bone in the metaphysis, in comparison to *STZ* , which showed less trabecular bone (Fig. *1B-E*, and yellow arrowheads), *STZ+SostAb* also appeared to have a more organized growth plate (Fig. *1D,E*). *SostAb* treatment dramatically increased trabecular bone formation in the epiphysis (Fig. *1F-H*); however, it did not rescue the increased adipogenesis seen in the marrow of *STZ+SostAb* mice (Fig. *1E*, black arrowheads). Cortical bone was much thicker in *SostAb* treated groups (Fig.*1J-M*, arrows), and while *STZ* mice show residual cartilage matrix in the cortical bone (Fig. *1L*, arrowhead) *SostAb* treatment of *STZ* mice resulted in less persistent cartilage (Fig. *1M*).

STZ mice had significantly reduced bone volume to total bone volume (BV/TV) ratio (p<0.02), connectivity density (Conn. Dens.) (p<0.03), trabecular thickness (Tb. Th.) (p<0.01), and bone mineral density (BMD) (p<0.05) in lumbar vertebrae compared to *Control* mice (Table 1, see also Fig.*1N,P*). In cortical bone, bone area: total area (BA/TA) (p<1x10^{-5}), bone area (BA) (p<5x10^{-5}), total area (TA) (p<0.01) and BMD (p<0.02) are reduced in *STZ* mice compared to *Control* (Table 1, see also Fig.*1R,T*). *STZ* mice also had a higher structural model index (SMI, plate model) (p<0.005). No changes were identified in trabecular number (Tb. N.) or trabecular separation (Tb. Sp.). *SostAb* treated mice had increased BV/TV (p<0.006), Conn. Dens. (p<0.05), and Tb. Th. (p<5x10^{-5}) compared to *Control* mice in L4 vertebrae (Table 1, see also Fig.*1N,O*). In cortical bone, *SostAb* mice have increased BA/TA (p<5x10^{-6}) and BA(p<0.001) compared to *Control* (Table 1, see also Fig. *1R,S*). In *STZ+SostAb* treated mice, BV/TV (p<2X10^{-5}), Conn. Dens. (p<1X10^{-4}), SMI (p<1X10^{-4}), Tb. Th. (p<1x10^{-7}), Tb.Sp. (p<0.05) are all improved compared to *Control*, and furthermore, BV/TV (p<1x10^{-6}), SMI (p<1x10^{-5}), Tb.N (p<0.02), Tb. Th. (p<2x10^{-8}), and Tb. Sp.(p<0.005) are improved compared to *STZ*-treated mice (Table 1, see also Fig. *1N,Q*). In cortical bone, *STZ+SostAb* mice have improved BA/TA (p<1x10^{-5}), BA (p<5x10^{-5}), TA (p<0.05), and BMD (p<0.01) compared to *STZ*-treated mice, and are similar to *Control* (Table 1, see also Fig. *1R,U*).

Improved STZ Bone parameters persist 3-weeks after discontinuation of SostAb treatment
To examine longer-range effects of *SostAb* treatment on improvement of diabetic bone, we examined bone parameters in 14-week old uninjured cohorts, after discontinuation of *SostAb* treatment. *STZ* mice had significantly reduced trabecular bone parameters compared to Control: BV/TV (p<0.02), Conn. Dens. (p<0.02), Tb N. (p<0.02),Tb. Th. (Tb. Th.) (p<0.05), and Bone Mineral Density (BMD) (p<0.05) in lumbar vertebrae compared to *Control* mice (Table 1, see also Fig.*1N,P*). *STZ* mice also have a higher Structural Model index (SMI, plate model) (p<0.02) and Tb. Sp. (p<0.03). In cortical bone, BA/TA (p<0.005), BA (p<0.0005), and TA (p<0.002) are reduced in *STZ* mice compared to *Control* (Table 2). No changes were identified in cortical BMD. *SostAb* treated mice have increased BV/TV (p<1x10^{-10}), Tb N. (p<5x10^{-6}) and Tb. Th. (p<1x10^{-10}) compared to *Control* mice in L4

vertebrae (Table 2). *SostAb* mice also have decreased SMI ($p<1x10^{-8}$) and Tb. Sp. ($p<2x10^{-6}$). No changes were identified in Conn. Dens. or trabecular BMD. In cortical bone, *SostAb* mice have increased BA/TA ($p<0.005$), BA ($p<0.0005$), and TA ($p<0.002$), but not BMD, compared to *Control* (Table 2). In *STZ+SostAb* treated mice, all trabecular parameters are improved, compared to *Control*, except trabecular BMD; BV/TV ($p<2x10^{-7}$), Conn. Dens. ($p<0.05$), SMI ($p<1X10^{-6}$), Tb. Th. ($p<3x10^{-7}$), Tb.Sp. ($p<2x10^{-5}$) indicate more trabecular bone. Additionally, BV/TV ($p<2x10^{-7}$), Conn. Dens. ($p<0.05$), SMI ($p<1x10^{-6}$), Tb.N ($p<3x10^{-5}$), Tb. Th. ($p<3x10^{-7}$), Tb. Sp.($p<0.2x10^{-5}$) and BMD ($p<0.03$) are improved compared to *STZ*-treated mice (Table 2). In cortical bone, *STZ+SostAb* mice have improved BA/TA ($p<0.0003$) compared to *Control*, and improved BA/TA ($p<1x10^{-5}$), BA ($p<5x10^{-5}$), and TA ($p<0.05$), compared to *STZ*-treated mice; cortical BMD is similar to *Control* and *STZ* (Table 2).

SostAb treatment improves bone parameters in fracture callus and intact bones of STZ-treated mice.

At 21 days post fracture, all groups had some amount of persistent cartilage in the mid-callus regions examined (Fig. 2A, *2B-E*). Both the *SostAb* and *STZ+SostAb* groups appeared to have more mineralized bone within the callus at 21 days compared to *Control* and *STZ* groups (Fig. *2B-E*). At 42 days post fracture, bridging had occurred in all groups at the fracture site, and some remodeling had occurred, as indicated by reduced trabecular bone in the callus region (Fig. *2F-I,* compared to *2B-E*). There appeared to be more marrow space at the bridging site in *STZ* mice compared to *Control*, and abundant adipocytes were still evident (Fig. *2F,H,* and arrowheads), while both *SostAb* groups appeared to have a more bone at the site of fracture compared to *Control* and *STZ* respectively (Fig. *2G,I*).

Representative μCT reconstructions of 21 day calluses are shown in Fig. *2K-N*. Reconstructions are consistent with histology, indicating a more porous bone structure in 21 day calluses of *STZ* mice compared to all other groups (Fig. *2M*). *SostAb*-treated groups (Fig. *2L,N*) appear to have thicker trabecular structure in calluses compared to *Control* and especially *STZ* mice (Fig. *2M*). Quantitative callus analysis by μCT confirmed the histology (Table 3). Compared to *Controls*, *STZ* calluses at 21 days have much decreased BV ($p<0.02$) and TV ($p<0.03$), indicating smaller fracture calluses, but this results in similar BV/TV ratios between the two groups. *STZ* calluses at 21 days also have reduced BMD compared to *Controls* ($p<0.01$). *SostAb* treated animals have higher BV/TV in the callus ($p<0.0005$), resulting primarily from a much larger BV value ($p<0.05$), since TV values do not differ from *Controls*. BMD in SostAb treated animals is actually reduced, compared to Controls ($p<0.01$), indicating that the bone is less mineralized. In *STZ+SostAb* treated animals, BV/TV is increased ($p<0.04$), TV is reduced ($p<0.04$) and BMD is reduced ($p<0.01$) compared to *Controls*, but BV/TV($p<0.02$) and BV ($p<0.05$) are significantly increased compared to *STZ* mice that received no antibody.

By 42 days post-fracture,*STZ* calluses had significantly less BV ($p<0.02$) but *SostAb* and *STZ+SostAb* groups had significantly more BV ($p<0.02$ and $p<0.01$, respectively) compared to *Controls*. *STZ+SostAb* treated animals at 42 days post-fracture retain their significant gains in BV ($p<0.001$), TV ($p<0.05$), and BV/TV($p<0.02$) compared to the diabetic *STZ* mice without antibody. However, *STZ* calluses at 42 days were only significantly different from *Controls* in BV parameters, indicating that 42 day calluses in *STZ+SostAb* mice were remodeling normally.

STZ-treated mice have impaired osteogensis during fracture repair

SP7/Osterix, an early osteoblast marker (Fig. *3A-D*), appeared dramatically elevated at 21 days post fracture in *STZ* calluses (Fig. *3C, arrows*), with large numbers of positive cells aligned on the bone surface, suggesting that lineage commitment and early osteoblast differentiation was not inhibited in *STZ* mice. However, Runx2, a pro-osteogenic transcription factor that is increased early in the

osteogenic differentiation program (Fig. *3E-H*), was reduced in *STZ* mice compared to *Controls* (Fig. *3E,G*), consistent with published results (López-Herradón, et al., 2013). *SostAb* treatment does not appear to impact levels in either *SostAb* mice, which have Runx2 levels that look similar to *Controls*, (Fig. *3E,F*) or *STZ+SostAb* calluses, which appear to have levels similar to or slightly increased, compared to *STZ* (Fig. *3G,H*). Collagen I, a matrix protein secreted by osteoblasts (Fig. *3I-L*) is reduced in *STZ* calluses (Fig. *3K*), which indicated a decrease in mineralized matrix production consistent with delayed or impaired osteogenesis, while *SostAb* and *STZ+SostAb* calluses showed increased levels (Fig. *3J,L*), compared to *Controls* (Fig. *3I*). Osteocalcin (Fig. *3M-P*) and DMP1 (Fig. *3Q-T*), markers of osteoblast maturation and osteocyte differentiation, appeared to have dramatically reduced signal in *STZ* calluses (Fig. *3O,S*), which indicated delayed callus maturation in *STZ* calluses, while *Control* (Fig. *3M,Q*), *SostAb* (Fig. *3N,R*), and *STZ+SostAb* (Fig. *3P,T*) groups revealed abundant signal along the surface of the bone. To determine if there is an alteration of cell apoptosis, Activated Caspase 3 antibody, an apoptosis marker (Fig. *3U-X*), was stained on 21 day callus sections, revealing no change in signal between any of the groups. This suggests that cell apoptosis was not contributing to the phenotype of *STZ* calluses at 21 days post-injury. At 21 days, *STZ+SostAb* calluses appeared to show patterns for all markers similar to *Controls* or *SostAb* calluses, rather than *STZ* calluses.

By 42 days after injury, the callus had matured in *Control* animals, and SP7/Osterix was still localized at the bone surface (Fig. *4A*). Collagen I was more abundant compared to 21 days (Fig. *3I,4E*), and DMP1 protein was plentiful in *Control* calluses (Fig. *4I*). Compared to *Controls*, *STZ* calluses continued to have an abundant number of SP7/Osterix –positive cells lining the bone surface, although not as plentiful as at 21 days (Fig. *3C,4C). STZ* calluses continued to have much reduced signal for Collagen I compared to *Controls* (Fig. *4E,G*), which suggested a continued delay in mineralization of the callus. However, by 42days post-injury, cells that became embedded in bone matrix of *STZ* calluses produced DMP1, at similar levels compared to the other groups (Fig. *4I-L*). Antibody-treated groups at 42 days post-fracture appeared to have more Collagen I in the callus relative to *Controls* or *STZ* calluses (Fig. *4E-H*), which indicated possible enhanced mineralization. There appeared to have been little impact of antibody treatment on the abundance of DMP1 in the bony calluses of *SostAb* or *STZ+ Sost Ab* groups, as all groups appeared to have similar levels (Fig. *4I-L)*.

While we did not see evidence of increased osteoblast apoptosis, we considered that enhanced osteoclast activity in *STZ* diabetic bones and could contribute to fracture callus instability and poor bone structure. We performed osteoclast-specific tartrate-resistant acid phosphatase (TRAP) stain on uninjured bones, and did not find any evidence of increased osteoclast activity with this marker in the growth plate (Sup. Fig. *1A-D*) or epiphyseal trabecular bone (Sup. Fig. *1E-H*), of *STZ* mice, nor did we see a difference with Cathepsin K antibody stain, which was used to examine osteoclast maturity in epiphyseal bone (Sup. Fig. *1I-L*). These data indicated that there is no difference in osteoclast activity of *STZ* diabetic mice, 5 weeks after onset of diabetes (at 11 weeks of age), and are consistent with other reports of normal osteoclast activity in T1DM (Motyl and McCabe, 2009; McCabe 2007; Hie et al., 2011)

Wnt signaling is diminished during diabetic fracture healing, and reversed with SostAb administration

To examine Wnt signaling activity we measured activated β-Catenin levels. In 21day calluses, there was no apparent activated β-Catenin signal in the bone of the callus of *STZ* treated mice (Fig. *5C*). *Control* calluses showed modest levels of activated β-Catenin, primarily localized to the bone-forming surface, with very few positive cells embedded in the bone matrix (Fig. *5A*). However, both antibody treated groups showed higher levels of activated β-Catenin at the bone surface, along with several positive cells in the bone matrix (Fig. *5B,D*), suggesting enhanced β-catenin activity in osteocytes as

well as in osteoblasts. By 42 days after injury, we found moderate levels of activated β-Catenin in the bone of *Control* mice, and it was no longer confined to the bone-forming surface (Fig. *5I*). *STZ* mice appeared to have small patches of activated β-Catenin, but the signal was primarily absent from osteocytes in the bone matrix (Fig. *5K*). In *SostAb* and *STZ+SostAb* treated mice, 42 day calluses showed robust activated β-Catenin signal throughout the callus (Fig. *5J,L*), which was increased over the levels seen in either *Controls* or *STZ* calluses (Fig. *5I,L*). These data demonstrated that *SostAb* administration elevated β-Catenin activity, consistent with Sost's role as a Wnt antagonist (Ellies at al., 2006).

Sclerostin levels were also examined in the callus;.at 21 days, we saw modestly increased Sclerostin in matrix-embedded cells of *STZ* mice, compared to *Controls*, consistent with expression in osteocytes (Fig. *5E,G*). We were, however, surprised to find dramatically increased sclerostin protein localized in the bone of *SostAb* treated groups (Fig. *5F,H*), in contrast to the enhanced activated β-Catenin activity we saw in these same groups (Fig. *5B,D*). By 42 days, we continued to find increased sclerostin signal in *STZ* calluses compared to *Controls* (Fig. *5M,O*). We also found further increased sclerostin localization in *SostAb* and *STZ+SostAb* treatment groups (Fig. *5N,P*). These findings were supported by an elevated level of Sost in the serum. *STZ* mice had a 30% increase in circulating Sclerostin, relative to *Controls* ($p<0.03$) (Fig. *5Q*). Circulating levels for sclerostin in *Controls* were within the reported range for the assay. Astonishingly, we found 8 times the amount of circulating sclerostin in both antibody-treated groups compared to *Controls* (*SostAb* $p<5\times10^{-9}$, *STZ+SostAb* $p<2\times10^{-7}$). These data indicated that sclerostin levels were increased in *STZ* diabetic animals, and abundant sclerostin protein was present in the bone tissue and in circulation of animals treated with *SostAb*.

Discussion:

In this study, we treated fractured bones in Type I diabetic mice with *SostAb* to improve fracture healing outcomes. We applied a clinically-relevant scenario to the administration of *SostAb*, to examine the effects on bone during fracture repair and after drug withdrawal, as it is unlikely that patients in a clinical setting would be treated indefinitely. We utilized a chemically-induced *STZ* diabetic model, to cause early-onset diabetes in growing mice. In this study, we found that even at these young ages, *STZ* diabetic mice lose bone mass and quality within 3 weeks of becoming diabetic. Although our model used young mice, our results are consistent with published reports of osteopenia in other *STZ* diabetic models (Musmeci et al., 2011; Lozano et al., 2011). The loss of bone we report here is primarily based on reduced bone formation, as we saw reduced mineral deposition, which was confirmed by immunofluorescence for matrix markers and uCT, and we did not find evidence of enhanced osteoclast activity in our model.

We found that *SostAb* treatment of *STZ* mice dramatically enhances bone formation, but depite the dramatic increases in bone volume, mineral density did not increase compared to *Controls*, suggesting that *SostAb* in the diabetic setting may function to promote osteoblast differentiation or osteoblast activity, rather than mineralization *per se*. Similarly, recent reports have demonstrated similar or reduced mineral content of bone after Sost antibody treatment, or due to genetic loss of Sost, despite abundant bone formation (Ross et al., 2014; Hassler et al., 2014). It is likely that reduction of Sost *via* antibody treatment increases osteoblast activity to produce abundant osteoid and undermineralized bone, which then mineralizes at a normal rate. Furthermore, early reports successful Sost antibody treatment to promote fracture healing relied heavily on models that primarily healed by intramembranous bone formation (Ominsky et al., 2011; McDonald et al., 2012; Jawad et al., 2013; Hamann et al., 2013). This suggested that *SostAb* may be beneficial in problematic fracture healing that results from poor osteogenesis; however, Li et al (2011) reported fracture repair in an endochondral model in *Sost*$^{-/-}$ mice, and Feng et al (2015) reported similar results with Sost antibody treatment, suggesting that fracture repair may benefit from early intervention by reducing

sclerostin levels, rather than only during osteogenesis. While we found a significant benefit in callus BV and BV/TV of *STZ+SostAb* mice, we also saw an increase in callus size, suggesting a benefit in early repair events prior to osteogenesis.

The large number of osteoblasts and precursors at the bone surface that express Sp7/Osterix, in the absence of significant mineral, suggests that the osteopenia in *STZ* diabetic mice is due to inability of osteoblasts to mature and produce significant mineral matrix. In another report, *STZ* mice had the same amount of immature mesenchymal tissue but decreased osteoblast differentiation. This was associated with decreased collagen type1, and Runx2 mRNA expression in an intramembraneous bone repair model (Lu et al., 2003), similar to our findings. Song et al (2012) demonstrated that loss of β-catenin in osteoblast precursors results in a switch from osteogenesis to adipogenesis that causes low bone mass. As our *STZ* mice have increased adiposity in the marrow, this is a plausible explanation for impaired osteogenesis in our *STZ* mice. Sost is not known to regulate the cell fate of precursor cells; in our study, SostAb appears to be unable to rescue this cell fate decision as *STZ+SostAb* mice still retain increased adiposity in the marrow. Thus, the cell fate decision in these osteoblast precursors is likely skewed by some influence other than the high circulating Sost levels seen in our *STZ* mice.

Another *STZ* diabetic model showed that in T1DM, insulin and IGF-1 deficiency decreases osteogensis due to negative regulation of Wnt signaling activity (Hie et al., 2011). It was demonstrated that the phenotype was caused by reduced levels of activated β-catenin, precipitated by a loss of phosphorylated GSK3β, and phosphorylated Akt/PKB, targeting β-catenin for degradation. They also observed a dramatic increase in Wnt inhibitor (Sost and Dkk1) levels. Loss of phosphorylated Akt/PKB can occur from a cycle of reduced Wnt signaling, as in the presence of high Sost levels, or in the event of oxidative damage. As a result, cells (such as osteoblast precursors and early osteoblasts) in a low insulin, high Sost, high glucose (oxidative) environment may be subject to cell cycle arrest (Liang and Slingerland, 2003). Another study observed a decrease in cyclin D mRNA expression in osteoblasts cells treated in a high glucose environment (Ma et al., 2014), which correlated with increased apoptosis and reduced cell proliferation. Taken together, these findings may account for the reduced number of Runx2-positive cells, and/or the apparent inhibition of differentiation of Osterix-positive cells seen in our *STZ* mice. While we did not observe enhanced activated caspase-3-driven apoptosis in calluses at 21 days, it is possible that early osteoblast or precursor death at another time point may contribute to reduced osteognesis in our T1DM model.

Reports are conflicting on whether T1DM induces changes in osteoclast activity, with some studies reporting enhanced, unchanged, or even reduced osteoclast activity in the context of T1DM. Reports of osteopenia compared to osteoporosis in T1DM are difficult to deconvolute, and are likely due to differences in age of animals or patients, time since diagnosis, diabetic complications (such as vascular or kidney disease), and body condition (Vestergaard et al., 2009; Hofbauer et al., 2007; McCabe, 2007). We found no change in osteoclast activity, which is consistent with the recent time since diagnosis and young age of our study groups.

Oxidative stress in tissues leads to much of the destructive sequelae in diabetic patients, causing pancreatic beta-cell death, kidney disease, cardiovascular disease, and may contribute to the effects of diabetes seen in the skeleton. Restoration of normal glucose levels does not prevent oxidative stress in tissues (Erejuwa, 2012). Reduced Wnt signaling and increased Wnt antagonism have been associated with increased oxidative stress, and reduced osteoblast activity (Hie et. al., 2011; Manolagas et al., 2007; Gaudio et al., 2012). Oxidative stress activates FOXO signaling, which competes for β-catenin in the cell, to reduce activation of Wnt target genes. Activation of Wnt signaling activates the Akt/PKB pathway; PKB then phosphorylates and sequesters FOXO in the cytoplasm, and prevents it from competing with β-catenin (Abiola et al., 2009). As Sost is an

antagonist of β-catenin-mediated Wnt signaling, reduction of Sost through SostAb activates β-catenin signaling, potentially mitigating the effects of oxidative stress and competition by activated FOXO (Manolagas et al., 2007). Thus, Wnt signaling may be suppressed by a mechanism that is independent of Sost, but can be mitigated by promoting Wnt signaling through inhibition of Sost. In this way, *SostAb* can alleviate the effects on diabetic bone that cannot be overcome by treatment with insulin.

We examined Sclerostin and β-catenin levels in our mice, to demonstrate that *SostAb* is effective at suppressing Sclerostin activity and elevating Wnt signaling activity through activation of β-catenin. We found elevated Sclerostin in serum, and decreased activated β-catenin in *STZ* mice, consistent with other reports of diabetic animals and patients with elevated Sclerostin levels (Hie et al., 2011; Manolagas et al., 2007; Iitsuka et al., 2013; Gennari et al., 2012). While we did find elevated β-catenin in antibody-treated groups, consistent with elevated Wnt signaling and promotion of osteogenesis, we also saw an unexpected and very highly elevated level of Sclerostin protein in the bone and blood of antibody-treated animals. This finding was unexpected, but there has been a recent report of Sclerostin-antibody treatment associated with elevated expression of Sclerostin (Stolina et al., 2014). At these extreme levels of Sost protein we find in the serum and bone, however, it is likely that the majority of the protein is inactivated by antibody bound to it. Sost antibody has been reported to have a very long lifespan in circulation; Ominsky et al (2010) reported Sost antibody levels in circulation of Cynomolgus monkeys and found that a single dose can be detected incirculation up to nearly a month post-dose,while Padhi et al. (2011) reported a single dose detected in serum up to an amazing 85 days after a single dose. This reveals some questions that remain unanswered: *SostAb* bound to Sclerostin may have a very long lifespan in tissues and doses of antibody given to be therapeutic may need to be relatively high to combat elevated expression and protein levels in response to treatment. Furthermore, sclerostin bound to *SostAb* may prevent degradation of either molecule, revealing a need to study downstream effects of this protein construct which appears to be in circulation for extended periods of time.

Our study has revealed that anabolic doses of *SostAb* enhance bone formation and quality in an early-onset Type I diabetic fracture healing model. We provide evidence that *SostAb* acts to promote osteoblast differentiation and alleviates inhibition on osteoblast differentiation caused by the diabetic state. We further demonstrated that the effects of *SostAb* treatment may extend well beyond the end of the dosing regimen, since our result show continued improvement of bone parameters in STZ diabetic mice treated with antibody, even three weeks after treatment had stopped. We also uncovered an as yet undocumented phenomenon of apparent accumulation of Sclerostin and/or Sclerostin-antibody complexes as a result of treatment, which is worth further exploration. In summary, *SostAb* is an effective treatment to counteract the low osteogenesis that occurs in T1DM bone environments, and may provide extended benefits beyond the treatment span.

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Figure Legends

Figure 1. ***SostAb treatment Improves Low Bone Mass in STZ mice.*** A timeline of the STZ and/or SostAb treatment illustrated at what age the mice were treated, injured, and euthanized (A). STZ and STZ+SostAb groups were administered with STZ daily for 5 days at 6 weeks old, allocating time for diabetes to develop prior to injury. At 8 weeks old, controls, STZ, SostAb, and STZ+SostAb groups were injured. Those treated with SostAb were treated at 3, 7, 10, 14, and 21 days post injury and euthanized at 21 or 42 days post fracture. Uninjured age-matched mice were euthanized as uninjured controls. Histological sections and stain with Alcian Blue and Nuclear Fast Red (B-M). The growth plate (B-E), Epiphysis (F-I), and Cortex (J-M) represents the location of the cartilage, trabecular bone, and cortical bone respectively. Alcian Blue stains the cartilage in blue, as indicated by the yellow arrows (C, D, E). STZ mice showed some cartilage present also in the cortical bone (L, yellow arrow). White circles, indicated by black arrows (D, H, E) show adipocytes within the bone marrow. The cortical bone (J-M) shows degrees of cortical bone thickness (vertical arrows) in each group. Uninjured 11 week old mice were euthanized and the L4 vertebrae (N-Q) and distal femurs (R-U) were scanned for uCT. L4 vertebrae uCT scans reveals a visualized amount of trabecular bone is present within each group. A trasverse plan on the distal uninjured femurs visually provides the differences of relative cortical bone thickness among each treated group.

Figure 2. ***Histology and MicroCT reveals SostAb Enhances Endochondral Healing.*** Histological sections on 21 and 42 days post fracture (dpf) callus were all analyzed and observed at the mid regions (A). Calluses at 21 dpf (B-E) and 42 dpf (F-I) were sectioned and stained with Alcian Blue and Nuclear Fast Red. Alcian Blue stains the cartilage blue, easily observed at 21 dpf calluses (B-D). Adipocytes cells, indicated by black arrows (H, I) were observed in the bone marrow, easily seen at 42 dpf calluses. Calluses at 21 pdf were also scanned for uCT, providing a visual illustration of the amount of woven bone present in the callus (K-N).

Figure 3. ***SostAb Rescues Abnormal Osteoblast Maturation in STZ Observed by Immunofluorescence.*** Fluorescenct immunostains on 21 dpf callus sections were completed with the markers of interest. Secondary antibodies of either green (Alexa Fluor 488) or red (Alexa Fluor 594) were used for detection and DAPI stains the nuclei of cells blue. An early osteoblast marker, Osterix, showed osteoblast residing along the surface of the woven bone as indicated by white arrows (A-D). An early osteoblast transcription factor, Runx2, is shown in panel E-H. Collagen 1, a protein secreted by osteoblasts, was shown in green within woven bone in panel I-L. A late osteoblast differentiation marker, Osteocalcin, and osteocytes, DMP1, are represented in panels M-P and Q-T respectively. Apoptosis marker, Caspase 3, signal was shown along the woven bone and in the bone marrow in panel U-X.

Figure 4. ***SostAb Anabolic Effect During Repair Continued After Treatment Termination.*** Calluses at 42 dpf were stained with markers of interest by fluorescence immunohistochemistry. Secondary antibodies of Alexa Fluor 488 (green) or Alexa Fluor 594 (red) were used for detection and DAPI stained the nuclei of cells blue. Osterix, an early osteoblast marker, signal was along the surface of the bone as indicated by the white arrows (A-D). Collagen 1, a protein secreted by osteoblasts, appeared within the bone in panels E-H. DMP1, an osteocyte marker, also appeared within the remodeling bone in panels I-L.

Figure 5. ***Immunohistochemistry Reveals Altered Wnt Signaling in Treated Groups.*** Fluorescence immunohistochemistry on beta-catenin was stained on 21 dpf calluses (A-D) and 42 dpf calluses (I-L). Alexa Fluor 594 (red) was used as a secondary antibody while the cells nuclei was stained with DAPI. Beta-catenin signal is initially expressed along the surface of the bone (A-D) and later within osteocytes during the remodeling stage (I-L). Sost antibody immunohistochemistry on 21

dpf calluses (E-H) and 42 dpf calluses (M-P) is expressed with the woven bone and osteocytes during the later stages of healing. Sost serum levels in each treated groups was measured using an ELISA kit for mouse Sost (Q) and represented in ng/ml. P-value scores of <0.05 are indicated by the asterisks.

Supplementary Figure 1. ***Osteoclast Activity was Unchanged in Treated Groups.*** Histological sections on uninjured 11 week old mice femurs were stained with TRAP, staining osteoclasts red and counterstained with Fast Green, staining bone blue (A-H). Analysis on osteoclast activity was observed at the growth plate (A-D) and epiphysis (E-H), representing the regions of natural osteogenesis and trabecular bone respectively. No alterations in osteoclast activity was observed, which was confirmed from fluorescence immunohistochemistry on cathepsin K (I-L) analyzed on trabecular regions just below the growth plate. Expression was shown by secondary antibody Alexa 594 (red) along the surface of trabecular bone.

Supplementary Methods

Histological sections on uninjured 11 week old mice femurs were de-waxed and stained with TRAP working solution for 1 hour at 37°C in the dark and counterstained with 0.02% Fast Green and mounted with 60% glycerol. Immunohistochemistry using primary antibody Cathepsin K (abcam, ab19027) required Uni-trieve (Innovex) for the antigen retrieval for 30 minutes at 65°C. Secondary antibody, Alexa Fluor 594 (red) Molecular Probes, were used for detection and mounted with Prolong Gold with DAPI (Molecular Probes). ImagePro Plus V7.0 and CCD QIClick camera were used for imaging.